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Static pressure accelerates ox-LDL-induced cholesterol accumulation via SREBP-1-mediated caveolin-1 downregulation in cultured vascular smooth muscle cells

Di-xian Luo^{a,b,c,1}, Cheng-lai Xia^{b,d,1}, Jun-mu Li^b, Yan Xiong^a, Hao-yu Yuan^{b,e}, Zhen-Wang TANG^b, Yixin Zeng^b, Duan-fang Liao^{b,f,*}

^a Department of Pharmacology, School of Pharmaceutics, Central South University, Changsha 410083, Hunan, China

^b Institute of Pharmacy and Pharmacology, College of Science and Technology, University of South China, Hengyang 421001, Hunan, China

^c First People's Hospital of Chenzhou City, Chenzhou 423000, Hunan, China

^d Department of Pharmacy, Third Affiliated Hospital Medical College of Guangzhou, Guangzhou 510150, Guangdong, China

^e Lusong Center for Disease Control and Prevention, Zhuzhou 412000, Hunan, China

^f Department of Traditional Chinese Diagnostics, School of Pharmacy, Hunan University of Chinese Medicine, Changsha 420108, Hunan, China

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ABSTRACT

Objective: To investigate the effect of static pressure on cholesterol accumulation in vascular smooth muscle cells (VSMCs) and its mechanism.

Methods: Rat-derived VSMC cell line A10 treated with 50 mg/L ox-LDL and different static pressures (0, 60, 90, 120, 150, 180 mm Hg) in a custom-made pressure incubator for 48 h. Intracellular lipid droplets and lipid levels were assayed by oil red O staining and HPLC; The mRNA levels of caveolin-1 and ABCA1, the protein levels of caveolin-1 SREBP-1 and mature SREBP-1 were respectively detected by RT-PCR or western blot. ALLN, an inhibitor of SREBP metabolism, was used to elevate SREBP-1 protein level in VSMCs treated with static pressure.

Results: Static pressures significantly not only increase intracellular lipid droplets in VSMCs, but also elevate cellular lipid content in a pressure-dependent manner. Intracellular free cholesterol (FC), cholesterol ester (CE), total cholesterol (TC) were respectively increased from 60.5 ± 2.8 mg/g, 31.8 ± 0.7 mg/g, 92.3 ± 2.1 mg/g at atmosphere pressure (ATM, 0 mm Hg) to 150.8 ± 9.4 mg/g, 235.9 ± 3.0 mg/g, 386.7 ± 6.4 mg/g at 180 mm Hg. At the same time, static pressures decrease the mRNA and protein levels of caveolin-1, and induce the activation and nuclear translocation of SREBP-1. ALLN increases the protein level of mature SREBP-1 and decreases caveolin-1 expression, so that cellular lipid levels were upregulated.

Conclusion: Static pressures stimulate ox-LDL-induced cholesterol accumulation in cultured VSMCs through decreasing caveolin-1 expression via inducing the maturation and nuclear translocation of SREBP-1.

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1. Introduction

Cholesterol accumulation in vascular smooth muscle cells (VSMCs) and monocytes/macrophages leads to the formation of li-

Abbreviations: VSMC, vascular smooth muscle cell; SREBP-1, sterol response element-binding protein-1; RT-PCR, reverse transcription polymerase chain reaction; QRT-PCR, quantitative reverse transcription polymerase chain reaction; ox-LDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified eagle medium; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; ALLN, N-acetyl-Leu-Leu-norleucinal.

* Corresponding author at: Department of Traditional Chinese Diagnostics, School of Pharmacy, Hunan University of Chinese Medicine, 300 Xueshi Road, Hanpu Science and Education District, Changsha 410208, Hunan, China. Fax: +86 0731 88458111.

E-mail addresses: luodixian_2@163.com (D.-x. Luo), dfliao66@yahoo.com.cn (D.-f. Liao).

¹ These authors contributed equally.

pid-loaded cells, causing a key atherogenesis event. Mechanical forces, such as shear stress and vertical pressure, are involved in cholesterol accumulation and even atherogenesis [1,2]. Hypertension is an independent risk factor for the development of atherosclerosis, although the mechanisms have not been well elucidated [3,4]. A popular evidence is that hypertension may exert oxidative stress on the arterial wall, which may contribute to atherosclerosis, fundamentally as an inflammatory disease [5]. Recently we also found that vertical static pressure may downregulate caveolin-1 expression in VSMCs cultured in vitro [6]. Caveolin-1, the most important member of the caveolin family with 21–24 kDa, is enriched in caveolae, and shuttles back and forth between the cytoplasm and cell membrane. Caveolin-1 plays an important role in intracellular cholesterol efflux. Caveolin-1-deficient L1210J cells cannot rapidly transfer intracellular synthetic cholesterol out of the cells [7]. The intracellular cholesterol

ester decreased 50% and the accumulation of cholesterol within the cell membrane increased in HepG2 cells transfected with caveolin-1 expression plasmid [8]. We also found in VSMC treated with 50 mg/L ox-LDL, caveolin-1 expression and accumulation within the cell membrane increased at the early stage, and decreased with time, especially when foam cells formed [9]. For the role of caveolin-1 in transmembrane transportation of cholesterol, in this study we clarified whether static pressure affects cholesterol transmembrane transport via regulating caveolin-1 expression.

Mechanical forces derived from blood flow include shear stress and vertical pressure. Shear stress regulates sterol regulatory element-binding proteins (SREBPs) activation and nuclear translocation via an integrin-Rho-ROCK-LIMK-Cofilin pathway [10]. However, it is not reported whether static pressure also activates SREBPs. SREBPs including SREBP-1a, SREBP-1c, and SREBP-2, play important roles in cellular sterol and lipid metabolism [11]. SREBP-1a and SREBP-2 are mainly involved in cholesterol metabolism [12], and SREBP-1c is engaged in fatty acid synthesis [13]. Inactivated SREBPs consist of a regulatory subunit and a DNA binding subunit, and are located in the endoplasmic reticulum (ER) membrane. When the intracellular sterol level is low, SREBPs are cleaved by S1P and S2P proteases to a regulatory subunit and a water-soluble N-terminal domain (the DNA binding subunit) [14], which is translocated to the nucleus to upregulate the expression of sterol biosynthesis-related genes including caveolin-1. In addition to being regulated by the content of cellular sterols, SREBP activation can be induced by shear stress, inflammatory cytokines [10,15]. Previous studies show that SREBP-1 inhibits caveolin-1 expression by binding SREs in the promoter of caveolin-1 gene [16,17]. Whether static pressure affects cholesterol accumulation by regulating caveolin-1 expression in VSMCs remains unclear. In this study, we investigated whether static pressures accelerate cholesterol accumulation via SREBP-1-induced downregulation of caveolin-1 expression in cultured VSMCs.

2. Materials and methods

2.1. Cell culture and pressure incubator

The pressure-adjustable cell incubator was custom-made as previously described [6]. The VSMC line (A10), derived from the thoracic aorta of embryonic rat, was cultured with DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO Life Technology, USA). VSMCs was used between passage 3 and 10. VSMCs at subconfluence of 50–60% was subjected to the experiments.

2.2. LDL isolation and oxidization

LDL was isolated from non-frozen human plasma according to the method described in our previous report [18,19]. In brief, the low density fraction was aliquoted into Quickseal tubes (Brown Caskets, Inc Miami, USA) after isolation of very low-density lipoproteins. The volume and the concentration of the solution in each tube were respectively adjusted to 35 ml and 1.063 kg/L with the buffer (38% NaBr and 0.15 M NaCl). The tubes were sealed and centrifuged at 130,000g at 4 °C for 20 h. Five microlitre LDL was recovered from supernatant in each tube. The isolated LDL was oxidized with 10 µmol/L CuSO₄ for 18 h at 37 °C. The oxidation degree of ox-LDL was assessed on the basis of increased mobility in agarose gel (compared to native LDL) and an increased level of thiobarbituric acid-reactive substances.

2.3. Oil red O staining

Cells were washed three times with cold phosphate-buffered saline (PBS), and then fixed with 10% formalin for 10 min. The for-

malin solution was removed and the cells were washed three times with cold PBS, and stained with oil red O solution (dissolved in isopropanol: water, 3:2) for 30 min, followed by washing with isopropanol for 15 s. Finally, the cells were stained with Hematoxylin and Eosin (HE) for 5 min to stain the nuclei, and images were captured at 20× magnification.

2.4. Rt-PCR

Total RNA was isolated from VSMCs with an RNeasy Mini Kit (Qiagen) and synthesized as cDNA with SuperScript III reverse transcriptase (Invitrogen). Caveolin-1 and GAPDH cDNA were amplified by PCR with DNA polymerase (Toyobo). The primer pairs used were as follows: human caveolin-1 (5'-TCTACAAGCCCAACAA-CAAGG-3' and 5'-ACCAACGACAGGAGTGAGAA-3', 920 bp), human ABCA1 (5'-CAGAGCCGAGCCGACCCTTC-3' and 5'-GCCTCCCAGGAGTCGGGTGA-3', 546 bp), and human GAPDH (5'-CGGAGTCAACGGA TTTGGTCGTAT-3' and 5'-AGCCTTCTCCATGGTGG TGAAGAC-3', 302 bp).

2.5. Quantitative PCR (qPCR)

The mRNA levels of ABCA1 and GAPDH were measured by real-time quantitative PCR. The primer pairs used were as follows: ABCA1 (5'-GCTCCTGCTGAAATACCG-3', and 5'-TGAGGGACGATTCC ACAT-3', 200 bp); GAPDH (5'-CGGAGTCAACGGATTGGT CGTAT-3' and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3', 302 bp). The threshold cycle (Ct) was defined as the cycle number at which a sample's fluorescence intensity (ΔR_n) crossed the determined threshold value reflecting a statistically significant point above the calculated baseline. Differences in the Ct values (ΔCt) between RhoA and GAPDH were calculated to determine the relative expression levels, using the following formula: $\Delta \Delta Ct = (\Delta Ct \text{ of pressure groups}) - (\Delta Ct \text{ of ATM group})$. The relative expression level between the groups was calculated according to the equation $2^{-\Delta \Delta Ct}$.

2.6. Lipid analysis by HPLC

Cellular free cholesterol (FC), and total cholesterol (TC) were analyzed by HPLC [19]. Briefly, VSMCs were placed into 0.9% NaCl solution, and homogenized on ice by sonication for 10 s. After the protein concentration was measured by bicinchoninic acid (BCA) assay (Pierce Biotechnology Company, USA), an equal volume of freshly prepared, cold (−20 °C) potassium hydroxide in ethanol (150 g/L) was added to the cell lysate and vortexed until clear. An equal volume of hexane isopropanol (v/v, 3:2) was added and vortexed for 5 min, and centrifuged at 800g for 5 min. The extraction procedure was repeated. The organic phase was transferred to glass tubes, and dried under nitrogen at 40 °C. The samples were dissolved in 100 µl isopropanol–acetonitrile (v/v, 20:80), followed by an ultrasound water bath at room temperature for 5 min. Finally, the samples were subjected to HPLC analysis (Agilent 1100, Agilent Technology, USA). Cholesterol was eluted with isopropanol–acetonitrile solution (v/v, 20:80) at the speed of 1 ml/min, and detected by 206 nm UV absorption.

2.7. Nuclear protein extract

VSMCs were washed three times with cold PBS, and scraped into lysis buffer (50 mM KCl, 25 mM Hepes (pH 7.8), 1 mM PMSF, 100 µM DTT, 10 µg/ml Leupetin, 25 µg/ml aprotinin, 0.5% Nonidet-P40 (NP-40)). Cell lysate was kept on ice for 15 min, and centrifuged at 420 g for 5 min. The pellet was washed once with washing buffer (50 mM KCl, 25 mM Hepes (pH 7.8), 100 µM DTT, 1 mM PMSF, 10 µg/ml Leupetin, 25 µg/ml aprotinin). Then extract

Table 1
Intracellular lipid content in VSMCs. VSMCs were treated with 50 mg/L ox-LDL and respectively with 0, 150 mm Hg and with or without ALLN for 48 h. Cellular lipid was extracted, and free cholesterol (FC), total cholesterol (TC) were analyzed by HPLC, CE and ratio of CE/TC were calculated.

Pressure (mm Hg)	FC (μg/mg protein)	CE (μg/mg protein)	TC (μg/mg protein)	CE/TC (%)
0	60.5 ± 2.8 ^{**}	31.8 ± 0.7 ^{**}	92.3 ± 2.1 ^{**}	34.5 ± 1.5 ^{**}
60	71.2 ± 3.4 ^{*,##}	51.6 ± 8.9 [*]	122.8 ± 12.3 [*]	42.0 ± 3.1 ^{*,##}
90	90.1 ± 7.1 [#]	73.7 ± 2.8 [#]	163.8 ± 9.9 [#]	45.0 ± 1.0 [#]
120	110.5 ± 18.1 ^{*,&}	135.1 ± 10.3 ^{*,***,&,&}	245.6 ± 7.8 ^{*,***,&,&}	55.0 ± 5.7 [#]
150	133.5 ± 11 ^{***}	184.4 ± 16.6 ^{*,***,###}	317.9 ± 5.0 ^{*,***,###}	58.0 ± 2.8 ^{***}
180	150.8 ± 9.4 ^{***}	235.9 ± 3.0 ^{*,***}	386.7 ± 6.4 ^{*,***}	61.0 ± 1.8 ^{***}

^{*} $p < 0.05$ vs. 0 mm Hg.
^{**} $p < 0.01$ vs. 90, 120, 150, 180 mm Hg.
^{***} $p < 0.01$ vs. 90 mm Hg.
[#] $p < 0.05$ vs. 60 mm Hg.
^{##} $p < 0.01$ vs. 150, 180 mm Hg.
^{###} $p < 0.01$ vs. 180 mm Hg.
[&] $p < 0.05$ vs. 180 mm Hg.
^{&&} $p < 0.01$ vs. 150, 180 mm Hg.

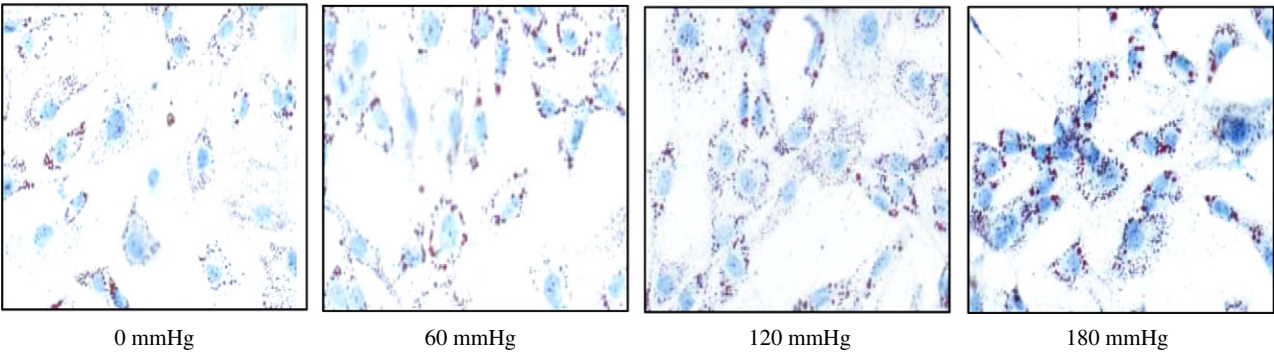
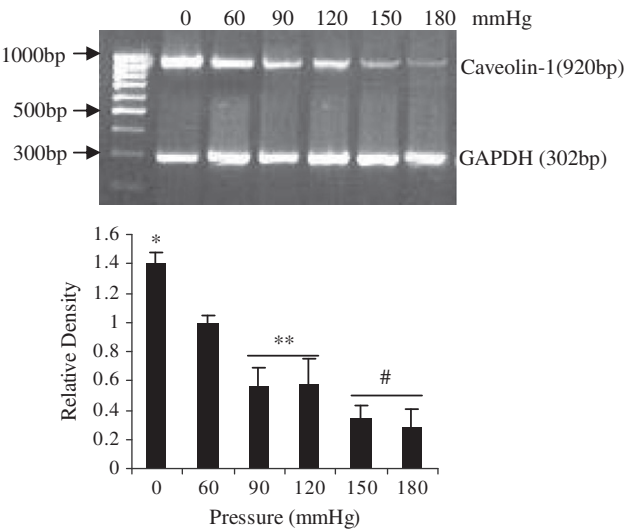


Fig. 1. Effect of static pressure on the levels of intracellular lipid droplets in VSMCs. 1×10^5 VSMCs were treated with 50 mg/L ox-LDL and respectively with 0, 60, 120, 180 mm Hg for 48 h. Then the cells were stained with oil red O and Hematoxylin and Eosin (HE). Images were captured at $20\times$ magnification.

buffer (500 mM KCl, 1.5 mM Hepes, pH 7.8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml Leupetin, 25 μg/ml aprotinin, 100 μM DTT, 10% Glycerol) was added into the tube containing

the pellet, and the tube was shaken for 30 min. Finally the tube was centrifuged at 20,800 rpm for 10 min, and the supernatant was kept as nuclear protein.

A. RT-PCR



B. Western blot

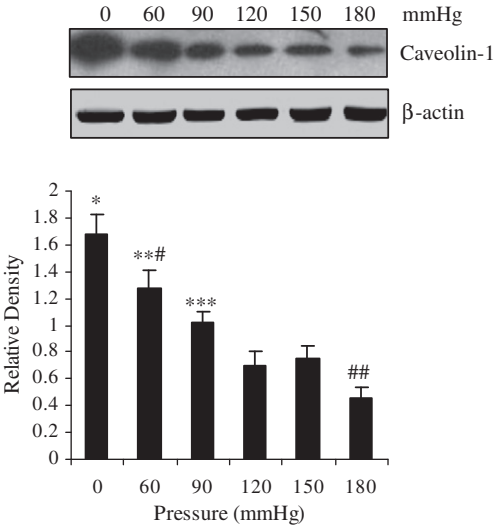


Fig. 2. Effect of static pressure on caveolin-1 expression in VSMCs. VSMCs were treated with 50 mg/L ox-LDL and respectively with 0, 60, 90, 120, 150, 180 mm Hg for 48 h. (A) Total RNA was extracted, and caveolin-1 mRNA level was detected by RT-PCR. Relative density means the ratio of caveolin-1 over GAPDH. ^{***} $p < 0.01$ vs other groups. (B) Total protein was extracted, and caveolin-1 protein level was detected by western blot. Relative density means the ratio of caveolin-1 over β-actin. ^{*} $p < 0.01$ vs 90, 120, 150, 180 mm Hg; ^{**} $p < 0.05$ vs 0, 90 mm Hg; ^{***} $p < 0.05$ vs 120, 150 mm Hg; ^{##} $p < 0.01$ vs 60, 90, 120, 150 mm Hg; [#] $p < 0.01$ vs 120, 150 mm Hg.

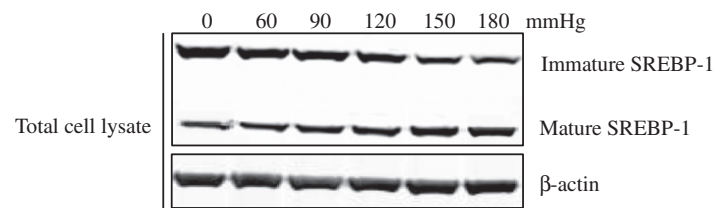
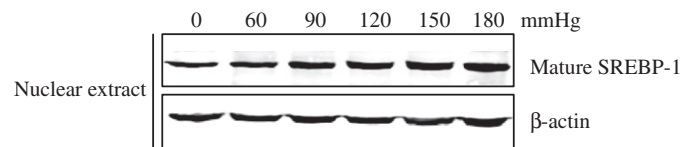
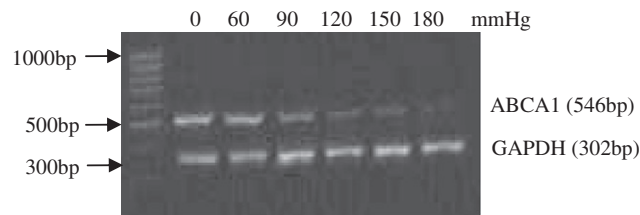
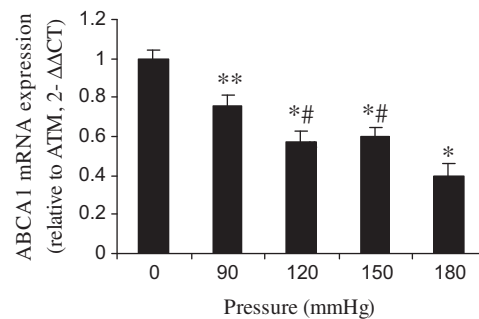
A. SREBP-1 in total cell lysate**B. SREBP-1 in nuclei****C. SREBP-1 activity in regulating ABCA1 transcription****i) RT-PCR****(ii) QRT-PCR**

Fig. 3. Effect of static pressure on SREBP-1 activation in VSMCs. VSMCs were treated with 50 mg/L ox-LDL and respectively with 0, 60, 90, 120, 150, 180 mm Hg for 48 h. (A) Total protein was extracted, and SREBP-1 was detected by western blot. (B) Nuclear protein was extracted, and mature SREBP-1 was detected by western blot. (C) SREBP-1 activity in regulating ABCA1 transcription. (i) RT-PCR, Total RNA was extracted, the mRNA level of ABCA1 was detected by RT-PCR as described in Methods section; (ii) QRT-PCR, The mRNA level of ABCA1 was quantified by QRT-PCR as described in Materials and Methods. * $p < 0.01$ vs ATM (0 mm Hg), ** $p < 0.01$ vs 180 mm Hg, # $p < 0.05$ vs 90, 180 mm Hg.

2.8. Western blot analysis

VSMCs were washed three times with cold PBS, and scraped into the lysis buffer (50 mM Tris-Cl, pH 8.0, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 5 mg/ml sodium deoxycholate) in the presence of 10 μ g/ml leupeptin, 0.5 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4 (Sigma, USA), followed by the centrifugation at 20,800g for 5 min. The supernatant was collected as total cell lysate. Protein concentration was measured with BCA assay, and then an equivalent amount of total protein from each sample was separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% fat-free dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) at room temperature for 1 h. The membrane was incu-

bated overnight with rabbit anti-caveolin-1 polyclonal antibody (1:1000), and mouse anti-SREBP-1 p68 monoclonal antibody (1:1000), anti- β -actin antibody (1:10,000) (Santa Cruz Biotechnology, USA), followed by the incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG (Boster Biotechnology, Wuhan, China) with the dilution rate of 1:1000 at room temperature for 1 h. Densitometry of images was scanned and analyzed with Alphamager 2200 Image software (UVP Company, USA).

2.9. Statistical analysis

Data represent means \pm s.d. from three independent experiments. Statistic analysis was performed using ANOVA test or Chi

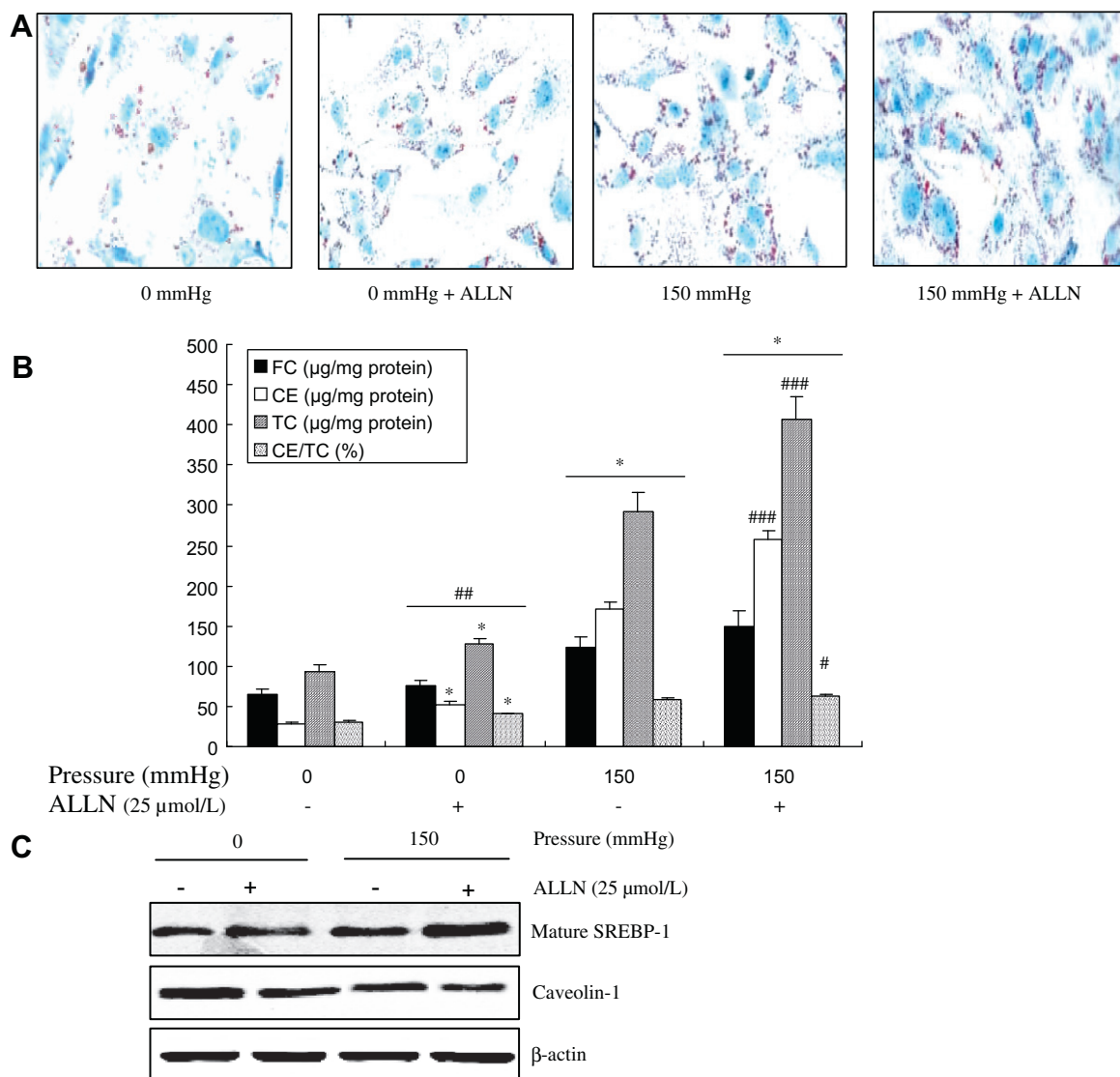


Fig. 4. Effect of inhibition of SREBP-1 catabolism on cholesterol accumulation and caveolin-1 expression in VSMCs. VSMCs were treated with 50 mg/L ox-LDL and respectively with 0, 150 mm Hg and with or without ALLN for 48 h. (A) The cells were stained with oil red O and Hematoxylin and Eosin (HE). (B) Cellular lipid was extracted, and free cholesterol (FC), total cholesterol (TC) were analyzed by HPLC, CE and ratio of CE/TC were calculated. * $p < 0.05$ and ** $p < 0.01$ vs 0 mmHg; ### $p < 0.01$ vs 150 mm Hg and 150 mm Hg with ALLN; # $p < 0.05$ vs 150 mm Hg; ### $p < 0.01$ vs 150 mm Hg. (C) Total protein was extracted from the cells, and mature SREBP-1 and caveolin-1 were detected by western blot.

square tests, as appropriate, with INSTAT statistical analysis package (Graph Pad Software, CA), for statistical significance at $p < 0.05$.

3. Results

3.1. Static pressure accelerated cholesterol accumulation in cultured VSMCs

To investigate the effect of static pressure on cholesterol accumulation in cultured VSMCs, 7.5×10^5 VSMCs were treated with 50 mg/L ox-LDL and different static pressures (0, 60, 90, 120, 150, 180 mm Hg) for 48 h. Total cellular lipids were extracted and measured by HPLC. As shown in Table 1, static pressures elevated cellular lipid content in a pressure-dependent manner, and intracellular free cholesterol (FC), cholesterol ester (CE), total cholesterol (TC) were respectively increased from 60.5 ± 2.8 mg/g, 31.8 ± 0.7 mg/g, 92.3 ± 2.1 mg/g at ATM to 150.8 ± 9.4 mg/g, 235.9 ± 3.0 mg/g, 386.7 ± 6.4 mg/g at 180 mm Hg. CE/TC rate reached more than 50% when static pressure ex-

ceeded 120 mmHg. 2.5×10^5 VSMCs were cultured overnight in 6-well plate, then treated with 50 mg/L ox-LDL and different static pressures (0, 60, 120, 180 mm Hg) for 48 h. The cells were stained by oil red O. As shown in Fig. 1, static pressures increased intracellular lipid droplets in a pressure-dependent manner, and the lipid droplets were the most when static pressure reached 180 mm Hg.

3.2. Static pressure downregulated caveolin-1 expression

For the important role of caveolin-1 in cholesterol efflux, we detected the mRNA and protein levels of caveolin-1 after static pressure treatment. Cells were treated with 50 mg/L ox-LDL and different static pressures (0, 60, 90, 120, 150, 180 mm Hg) for 48 h. Total RNA and cell lysate were extracted from the cells, and subjected to detect the RNA and protein level of caveolin-1 by RT-PCR or western blot. As shown in Fig. 2, static pressures induced caveolin-1 transcription and expression in a pressure-dependent manner with the lowest level at 180 mm Hg.

3.3. Static pressure induced SREBP-1 activation and nuclear translocation

It has been reported that SREBP-1 regulates the caveolin-1 expression. To examine the role of SREBP-1 under static pressure treatment, we determined the level of active SREBP-1 in total cell lysate and the nuclei by western blot. As shown in Fig. 3A and B, static pressures activate SREBP-1, and mature SREBP-1 was increased in a pressure-dependent manner. Static pressure also induced the nuclear translocation of SREBP-1. To further confirm the elevated biological activity of SREBP-1, we measured the mRNA level of ABCA1, one of important SREBP-1 target genes by common RT-PCR and quantitative real time RT-PCR. As shown in Fig. 3C, the mRNA level of ABCA1 was significantly downregulated in a pressure-dependent manner, consistent with the elevated SREBP-1 activation.

3.4. Inhibition of SREBP-1 catabolism aggravated downregulation of caveolin-1 expression and cholesterol accumulation

To further confirm the relationship between SREBP-1 and static pressure, VSMCs were treated with 50 mg/L ox-LDL and with or without 150 mm Hg or 25 μ mol/L ALLN for 48 h. Total cell lysate was extracted and subjected to oil red O staining and western blot. ALLN significantly increased intracellular lipid droplet (Fig. 4A), and cholesterol accumulation in VSMC (Fig. 4B). ALLN also promoted SREBP-1 maturation and decreased caveolin-1 expression (Fig. 4C), at both ATM and 150 mm Hg.

4. Discussion

Hypertension is an independent risk factor for the development of atherosclerosis, but the mechanism has not been well elucidated. Formation of VSMCs- or macrophage-derived foam cells is a key pathologic process in atherosclerosis. However, it is unknown how pressure affects the formation of foam cells. We investigated the effect of static pressure on cholesterol accumulation in VSMCs cultured in vitro. In this study, we found static pressure significantly increased the accumulation of FC, CE, TC in VSMCs in a pressure-dependent manner. VSMCs are named as lipid-loaded cells before the ratio of cholesterol ester in total cholesterol is less than 50%, or else named as foam cells if the ratio is more than 50%. Intracellular CE/TC rate exceeded 50% when VSMCs were treated with the pressure more than 120 mm Hg. Our research results are consistent with the previously described relationship between high blood pressure and atherosclerosis.

We also investigated the mechanism of the effect of static pressure on cholesterol accumulation in ox-LDL-incubated VSMCs. We found that static pressure decreased the expression of caveolin-1 in the pressure-dependent manner. Caveolin-1 is an important structure component of caveolae, a transport center of cholesterol [20]. Caveolae decrease if caveolin-1 expression is downregulated, so that cholesterol efflux also decreases, conversely cholesterol accumulation increases [8]. Additionally, caveolin-1 plays another role in cholesterol efflux by the forms of intracellular trafficking complex containing caveolin-1, HSP56, cyclophilin A, cyclophilin 40. Caveolin-1 complex presents cholesterol to the transmembrane transport systems of ABCA1 in the cholesterol transportation center of caveolae, and finally ABCA1 transports cholesterol to extracellular space [21,22]. SREBP-1 activity assay in this study showed that ABCA1 was downregulated by static pressure (Fig. 3C). Therefore, static pressure-induced downregulation of caveolin-1 expression resulted in cholesterol accumulation in VSMCs cultured in vitro.

We found that SREBP-1, a transcriptional regulation factor of caveolin-1, was activated by static pressure, which was confirmed by the experiment that the SREBP-1 level was increased by ALLN-mediated inhibition of SREBP-1 catabolism (Fig. 4C). Active SREBP-1 (p68) translocated into the nucleus to regulate caveolin-1 transcription because there are two SREs in the promoter of caveolin-1. The ER-to-Golgi translocation of SREBP-1 requires the actin-based cytoskeleton and is enhanced by shear stress-activated Rho-ROCK-LIMK-cofilin pathway. However, it is unclear whether static pressure-induced SREBP-1 activation is also mediated by the Rho-ROCK-LIMK-cofilin pathway. We will investigate whether the pathway is involved in pressure-induced SREBP-1 activation in the future.

In a word, static pressures stimulate ox-LDL-induced cholesterol accumulation in cultured VSMCs through caveolin-1 downregulation that is induced by the maturation and nuclear translocation of SREBP-1 in a pressure-dependent manner. The study not only shows another evidence that hypertension is an independent factor of atherosclerosis, and blood pressure leads to the formation of lipid-loaded or foam cells, but also provides targets for the prevention and treatment of atherosclerosis in hypertensive patients.

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